FACTORS AFFECTING GROWTH and SCLEROTIAL FORMATION OF *SCLEROTINIA MINOR* IN VITRO

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ABSTRACT. — Growth and sclerotial formation of three isolates of *Sclerotinia minor* were excellent on PDA, Carrot-agar, Glucose-NH_4NO_3-agar with Iron-minor element and Glucose-peptone-agar. Of the synthetic and semi-synthetic media tested, Glucose-peptone was optimal for growth and sclerotial formation. *S. minor* grew and produced sclerotia over a wide range of pH (2.5-8.0). An optimum pH of 4.0 was obtained for growth and sclerotial formation both in buffered and unbuffered media. The temperature range for good vegetative growth and sclerotial formation was from 15 to 26°C with an optimum at the latter temperature. At the maximum temperature of 30°C only vegetative growth occured. At 15°C sclerotia tend to unite and to become irregular and flattened, smaller than those produced at 25°C.

RÉSUMÉ. — La croissance mycélienne et la formation des sclérotes de trois isolats de *Sclerotinia minor* sont excellentes sur PDA, Carotte-agar, Glucose-NH_4NO_3-agar avec le fer comme microélément, et Glucose-peptone-agar. Ce dernier était le meilleur milieu parmi tous ceux qui ont été testés qu'ils soient synthétiques ou semi-synthétiques. *S. minor* croît et produit des sclérotes dans une large gamme de pH (2,5-8). Un optimum de pH 4,0 est obtenu pour la croissance et la formation des sclérotes en milieux tamponnés ou non. Les températures favorables à la croissance mycélienne et à la formation des sclérotes se situent entre 15 et 26°C avec un optimum à cette dernière température. A la température maximale de 30°C on observe seulement une faible croissance végétative. A 15° les sclérotes tendent à s'aggréger et prennent une forme plate à bords irréguliers. Leurs dimensions sont inférieures à celles des sclérotes obtenus à 25°C.

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INTRODUCTION

The occurrence of this small sclerotial fungus was first observed on lettuce in Massachusets by SMITH (1900) who considered it as a degenerate form of *Sclerotinia libertiana* Fuckel (= *S. sclerotiorum* (Lib.) De Bary). JAGGER (1920) studied various isolates of this fungus on the same host and described them as a new species, *Sclerotinia minor*. The pathogen has since been reported to occur on a number of plants (VIENNOT-BOURGIN, 1949; WALKER, 1952; SACKSTON, 1956; SALERNO, 1959; RAINBOW, 1970, etc...). *S. minor* Jagger has been considered to be a synonym of *S. sclerotiorum* (Lib.) De Bary by some authors (PURDY, 1955; WALKER, 1969; MORRALL et al., 1972). However, recent studies based on patterns of mycelial growth, sclerotial ontogeny, their soluble proteins and isoenzymes showed *S. minor* and *S. sclerotiorum* to be distinct from each other (cf. WILLETTS and WONG, 1971; WILLETTS, 1972; WONG and WILLETTS, 1973). In France, the pathogen has been found severely attacking lettuce crops, particularly in the regions of the eastern Pyrénées where losses up to 80 % have been recorded (WAFFELAERT, 1969).

Since the physiology of *S. minor* has not been previously investigated, with the exception of CHIVERS (1929), on the effect of temperature on the size of sclerotia, MARRAS (1961), the effect of temperature and pH on growth on carrot-agar and LOUVET and BULIT (1964), on the influence of different concentration of CO_2 on growth, the present work was undertaken to study nutritional requirements and various factors affecting growth and sclerotial formation of the pathogen. However, only results of the effect of different media, pH and temperature are reported in this paper.

MATERIALS AND METHODS

The three isolates of *S. minor* used in this investigation were originally isolated from diseased sunflower (Iran), lettuce (France) and lucerne (U.S.A.), and have been referred to as Sm-S, Sm-LT and Sm-LC, respectively. The first two isolates were obtained through the courtesy of Prof. G. VIENNOT-BOURGIN and the third one was kindly arranged for us by Prof. L.H. PURDY. Stock cultures used were maintained on PDA at 4°C and were transferred regularly at intervals of two months.

Isolates were grown on ten natural and synthetic solid media (20 ml/plate) prepared according to the formulae given by HAWKER (1950) except Glucose-NH_4NO_3-Iron-minor element (abbreviated as GAIME) and COON's medium which had the following compositions per litre of distilled water, GAIME : Glucose, 20.0g – NH_4NO_3, 1.0g – KH_2PO_4, 1.0g – $MgSO_4$ 7 H_2O, 0.5g – $FeSO_4$ 7 H_2O, 2 mg – $ZnSO_4$ 7 H_2O, 2mg – $CaSO_4$ 2 H_2O, 2 mg – $CuSO_4$ 5 H_2O, 1mg – $MnSO_4$ H_2O, 0.2mg – Na_2MoO_4 2 H_2O, 0.2mg; COON's medium : Maltose 3.5g – KH_2PO_4, 1.25g – $MgSO_4$ 7 H_2O, 0.5g – Asparagine

0.25g. Colony growth (average of two) and visual observation on sclerotial formation of each isolate were recorded on the third, fifth and tenth day after inoculation and incubated at 24 ± 2°C in continuous artificial light. The growth in terms of dry weight and sclerotial formation were compared on seven synthetic liquid media also. Isolates were grown on these media under similar conditions for 21 days.

The effect of pH on growth was studied both in unbuffered and buffered glucose-peptone medium (citrate-phosphate buffer : 0.01 M citric acid and 0.02 M disodium hydrogen phosphate, pH 2-8, with 0.5 of intervals). Isolates were grown on different pH at 25°C for 17 days in a stationary condition.

The effect of temperature on growth and sclerotial formation was investigated on PDA (20 ml/plate), incubated at various temperatures ranging from 0-35°C in dark. Visual observations on the degree of sclerotial formation, as in all the experiments, were empirically rated as nil, scanty, moderate or abundant (see table 1). Isolates were also grown in glucose-peptone medium at 5-30°C and incubated for 17 days under the same condition.

250 ml Pyrex Erlenmeyer flasks, each containing 50 ml of liquid medium were sterilized for 15 minutes at 115°C and inoculated with a 4 mm mycelial disc cut from the advancing margin of a 4 days old culture grown on PDA at 20°C. Experiments were run in triplicates. Unless otherwise mentioned, the pH of the media were adjusted to 6.0 with NaOH or HCl prior to autoclaving (recorded as initial pH) and at the end of incubation period.

Mycelial mats and sclerotia were harvested by filtration with suction through «Millipore» filter discs which had been previously dried to constant weight at 80°C. Filtrates from the replicates were mixed and the pH values were determined. Mycelium and sclerotia were thoroughly washed with distilled water, dried at 80°C for 24 hrs and weighed.

RESULTS

Effect of natural and synthetic / or semi-synthetic media

PDA, carrot-agar, malt-agar, glucose-peptone-agar and GAIME all provided very good growth and sclerotial formation of the three isolates (Table 1). The growth and the sclerotial formation was moderate in CZAPEK-DOX and RICHARDS'S agar, and poor growth with a few mature sclerotia occurred in ASTHANA and HAWKER's, BROWN's and COON's agar. However, sunflower isolate could not produce even a single sclerotium in ASTHANA and HAWKER's agar up to 10 days and lucerne isolate produced only few immature slcerotia in BROWN's agar. These isolates, however, produced moderate to abundant sclerotia on other media.

Isolates when grown in different synthetic and semi-synthetic media, produced maximum growth in glucose-peptone (Table 2). Good growth with abun-

Media	Initial pH	Sclerotia	Sm-LC			Sm-LT			Sm-S		
			3d	5d	10d	3d	5d	10d	3d	5d	10d
Asthana & Hawker's	5,7	immature	0 (60)*	0	0	0 (47)	+	0	0 (34)	0	0
		mature	0	0	+	0	0	+	0	0	0
Brown's	6,0	immature	0 (32)	0	+	0 (29)	+	0	0 (32)	0	0
		mature	0	0	0	0	0	+	0	0	0
Carrot - agar	5,8	immature	0 (62)	+++	0	0 (54)	+++	0	0 (47)	++	0
		mature	0	0	+++	0	0	+++	0	0	++
Coon's	5,9	immature	0 (32)	+	0	0 (21)	+	+	0 (12)	0	0
		mature	0	0	+	0	0	+	0	0	+
Czapek - Dox	5,8	immature	0 (62)	0	0	0 (42)	+	++	0 (40)	0	0
		mature	0	0	++	0	0	+	0	0	++
Glucose NH_4NO_3	5,7	immature	0 (60)	+	0	0 (45)	++	0	0 (41)	0	0
		mature	0	0	+++	0	0	+++	0	0	++
Glucose - peptone	5,6	immature	0 (62)	+	0	0 (41)	++	0	0 (39)	+	+
		mature	0	0	+++	0	0	+++	0	0	++
Malt - agar	5,9	immature	0 (63)	+++	+++	0 (46)	++	0	0 (42)	+	0
		mature	0	0	+++	0	0	+++	0	0	++
PDA	5,7	immature	0 (82)	++	0	0 (59)	++	0	0 (57)	+++	0
		mature	0	0	+++	0	0	+++	0	0	+++
Richards	5,8	immature	0 (50)	0	0	0 (40)	+	0	0 (38)	0	0
		mature	0	0	++	0	0	+	0	0	++

Table 1. — Sclerotial formation of the three isolates of *S. minor* (+ scanty; ++ moderate; +++ abundant) with immature sclerotia (initials and discolored sclerotia) and mature sclerotia (black) and radial growth (in mm*) on different solid media at 24 ± 2°C in continuous artificial light (Average of three replicates).

After 5 to 10 days the fungus spreads on the entire surface of the Petri dishes with the exception of the ASTHANA and HAWKER's, and BROWN's media.

dant to moderate sclerotia occurred in GAIME followed by RICHARDS's and CZAPEK-DOX, and poor growth with a few sclerotia in ASTHANA and HAWKER's, BROWN's and COON's media. Of the three, lucerne isolate produced maximum growth in all the media. At the end of incubation period, pH of all the media moved downwards.

Table 2. — Average dry weight in mg of mycelium and sclerotia[1] obtained by three isolates of *Sclerotinia minor* in different liquid media after 21 days of incubation at 24 ± 2 °C in continuous artificial light.

Media	initial pH	Sm-LC			Sm-LT			Sm-S		
		pH of filtr.	dry weight	scl	pH of filtr.	dry weight	scl	pH of filtr.	dry weight	scl
Asthana & Hawker's	5.7	3.4	37	+	5.5	8	0	3.5	25	+
Brown's	6.1	3.5	25	+	3.5	18	+	3.4	16	+
Coon's	5.8	5.6	10	+	5.5	11	+	4.2	21	+
Czapek-Dox	5.9	3.1	170	+++	3.2	154	+++	3.0	145	++
Glucose-NH_4NO_3	5.7	2.7	266	+++	2.8	222	+++	2.8	188	+++
Glucose-peptone	5.8	2.5	284	+++	2.6	228	+++	2.6	210	+++
Richards	5.8	3.0	178	+++	3.0	160	+++	3.2	131	++

1. Sclerotial formation : 0 nil, + scanty; ++ moderate; +++ abundant.

Effect of hydrogen-ion concentration

Table 3. — Effect of different pH on the growth[1] and sclerotial formation of three isolates of *S. minor* in buffered[2] and unbuffered liquid glucose-peptone medium after 17 days of incubation at 25 °C in dark and stationary condition.

	Sm-LC						Sm-LT						Sm-S					
	unbuffered medium			buffered medium			unbuffered medium			buffered medium			unbuffered medium			buffered medium		
	dry wt.	pH filtr.	scl	dry wt.	pH filtr.	scl	dry wt.	pH filtr.	scl	dry wt.	pH filtr.	scl	dry wt.	pH filtr.	scl	dry wt.	pH filtr.	scl
2.0	2	2.2	0	4	2.1	0	2	2.1	0	0	2.0	0	0	2.0	0	3	2.0	0
2.5	124	2.4	++	150	2.4	++	82	2.4	+	100	2.5	++	80	2.5	+	84	2.5	+
3.0	266	2.8	+++	276	3.0	+++	226	3.0	+++	242	3.0	+++	168	2.9	++	202	3.0	++
3.5	278	3.0	+++	332	3.4	+++	245	3.1	+++	267	3.5	+++	179	3.1	+++	250	3.5	+++
4.0	287	3.1	+++	360	3.8	+++	276	3.0	+++	288	3.7	+++	191	3.2	+++	266	3.7	+++
4.5	272	3.0	+++	354	3.8	+++	260	3.0	+++	278	3.1	+++	185	3.0	+++	275	3.1	+++
5.0	268	3.0	+++	301	4.0	+++	190	2.9	+++	264	3.4	+++	162	3.0	+++	248	3.4	+++
5.5	248	3.0	+++	262	3.6	++	177	2.9	+++	243	3.8	++	140	3.0	++	224	3.8	++
6.0	224	2.6	+++	245	3.8	++	159	2.7	+++	227	3.2	++	140	3.0	++	165	3.2	++
6.5	192	2.8	++	218	3.6	+	136	2.8	++	198	3.5	+	127	2.9	++	128	3.1	+
7.0	175	3.2	++	110	4.2	+	79	2.6	+	108	4.1	+	101	2.8	+	83	4.1	+
7.5	110	3.2	+	16	7.5	0	62	3.2	+	12	7.5	0	44	3.3	+	11	7.5	0
8.0	82	4.2	+	12	7.9	0	51	3.4	+	10	8.0	0	18	4.4	+	8	8.0	0

1. Average dry weight of mycelium and sclerotia in mg.
2. Citrate-phosphate buffer (0.01 M solution of citric acid, 0.02 M solution of dibasic sodium phosphate).
3. Sclerotial formation (see table 2).

Results of this experiment indicate that all the three isolates of *S. minor* grew and produced sclerotia within a wide range of pH (2.5-8.0) in an unbuffered medium (Table 3). The isolates grew well and produced many sclerotia between pH 3.0-6.0. Maximum growth in all the three isolates occurred at pH 4.0. On both sides of this optimum pH the growth decreased although the decrease was more abrupt towards the alkaline side than on the acidic side.

On buffered medium too, pH 4.0 was observed optimum for growth of all the isolates, except sunflower isolate which produced maximum growth at pH 4.5. However, there was no significant difference in growth produced at pH 4.0 and 4.5 by all the three isolates. The buffering maintained the pH during growth only from 2.0 to 3.5 and at 7.5 and 8.0. The formation of sclerotia at pH 7.5 and 8.0 was poor in all the isolates, none being in the buffered medium. No growth and sclerotial formation was observed at pH 2.0 regardless of the medium.

Effect of temperature

Isolates grew well and produced abundant slcerotia between 15-26°C (Table 4). Less growth with fewer or no sclerotia occurred at 5 and 28°C in all the isolates, except lucerne isolate which produced sclerotia moderately at the latter temperature. The optimum and maximum temperatures for growth of all the isolates were found to be 26°C and 30°C. At 30°C no sclerotium formed in all the three isolates.

In the case of solid medium the colony growth in all the isolates was inhibited after seven and four days at 0 and 30°C respectively, and no sclerotial formation

Table 4. — Effect of different temperatures on growth[1] and sclerotial formation of three isolates of *S. minor* in glucose-peptone medium after 17 days of incubation in dark and stationary condition (initial pH, 5.9).

Temperature	Sm-LC			Sm-LT			Sm-S		
	pH of filtrate	dry weight	scl[2]	pH of filtrate	dry weight	scl	pH of filtrate	dry weight	scl
5	3.1	57	0	3.0	64	++	3.6	29	0
15	2.6	178	+++	2.9	144	+++	3.0	102	++
20	2.8	182	+++	2.7	192	+++	2.6	184	+++
22	2.7	226	+++	2.6	232	+++	2.7	188	+++
26	2.8	260	+++	2.6	271	+++	2.5	225	+++
28	3.3	94	++	4.2	28	0	3.7	21	0
30	5.6	2	0	5.7	3	0	5.8	0	0

1. Average of dry weight of mycelium and sclerotial of three replicates in mg.
2. Sclerotial formation (see table 2).

was observed at these temperatures even after 30 days. At 35°C all the three isolates failed to grow. At 5 and 15°C sclerotial initial formed in ten and eight days respectively towards the periphery but the maturation of sclerotia was much delayed. At lower temperatures (5-15°C) sclerotia tended to unite, becoming somewhat irregular and flattened. Their size, however, was smaller than those produced at 25°C.

DISCUSSION

The results show that growth and sclerotial formation of *S. minor* was comparatively better in natural media than in other media tested. Of the liquid media, glucose-peptone supported maximum growth with abundant sclerotial formation. During the growth of isolates, the pH of the medium decreased and this decrease may be perhaps due to production of organic acids in the culture by the fungus as reported in case of *S. sclerotiorum* (Lib.) De Bary by VEGA et al. (1970).

The suitable pH range, 3.0-6.0 and the optimum pH 4.0 for growth and sclerotial formation of *S. minor* are contrary to MARRAS (1961) who obtained good growth of the fungus between pH 4.0 and 10.0 with the optimum around 7.0 on carrot-agar. In this study the fungus grew very little at pH 8.0. Incidentally the low pH of 3.5-4.5 which favours maximum growth and abundant sclerotial formation of *S. minor*, is also the best for the production of proteolytic enzymes (KHARE and BOMPEIX, 1976).

The minimum, optimum and maximum temperatures for growth of *S. minor* were observed to be 0, 26 and 30°C respectively whereas MARRAS (1961) reported them to be 5, 20-25 and 30°C on carrot-agar. However, in the present study, at 5 and 30°C only vegetative growth was observed, which was inhibited after seven and four days of inoculation at these temperatures. The size of sclerotium at low temperature (5°C) was found to be smaller than those produced at 25°C. This is in agreement with CHIVERS (1929) who also observed smaller sized sclerotia at low temperature.

Since *S. minor* is considered either synonymous to *S. sclerotiorum* by some authors or distinct by others it is necessary to compare our results of the effect of temperature and pH on growth and sclerotial formation of *S. minor* with that available for *S. sclerotiorum* in literature. However, comparisons between results of *S. minor* in this study and that of *S. sclerotiorum* (TANRIKUT and VAUGHAN, 1951) are difficult because of different media, incubation period and temperature. Nevertheless differences are evident, *S. minor* could not produce sclerotia at 0 and 30°C, at pH 2.0, and suitable pH range being 3.0-6.0 whereas TANRIKUT and VAUGHAN (1951) and LETOURNEAU (1979) reported that *S. slcerotiorum* produced slcerotia at these temperatures and pH, and grew well over a pH range of 2.4-9.6. However, the optimum temperature for this fungus as reported here and that of MARRAS (1961), and for *S. sclero-*

tiorum observed by TANRIKUT and VAUGHAN (1951) is more or less the same.

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